Research Paper:

The Nephroprotective Effect of Clove Oil (*Oleum Caryophylli*) Against Levofloxacin Toxicity in Rats

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ABSTRACT

Background: Levofloxacin is a fluoroquinolone antibiotic that has broad-spectrum antimicrobial activity, but it may induce kidney dysfunction. Clove oil (*Oleum caryophylli*) has antioxidant properties that may alleviate levofloxacin toxicity. This study aimed to examine the protective effect of clove oil on levofloxacin-induced nephrotoxicity in rat animal models.

Methods: A total of 24 male rats were divided into 6 groups. One group did not receive levofloxacin to serve as the control. The treatment groups received a single daily administration of levofloxacin (93 mg/kg) with either placebo or clove oil (10 mg/kg, 25 mg/kg, or 50 mg/kg per body weight) pre-treatment. Another group received Curcuma extract pre-treatment as a comparison. Blood samples were withdrawn after 28 days of treatment to measure serum biomarkers (urea and creatinine), and the kidneys were removed to measure renal Malondialdehyde (MDA) and histopathological analysis.

Results: The results showed that clove oil pre-treatment at a dose of 10 mg/kg was able to reduce renal MDA and serum biomarker levels (P<0.05). The effect was similar to that found in Curcuma-treated rats. In addition, clove oil (10 mg/kg) was also found to ameliorate renal histopathological damage due to levofloxacin.

Conclusion: Based on biomarker and histopathological analysis, clove oil pre-treatment in rats provides a nephroprotective effect against levofloxacin toxicity.

Keywords: Clove oil, Levofloxacin, Nephrotoxicity, *Oleum caryophylli, Syzygium aromaticum*

Introduction

Levofloxacin is a fluoroquinolone antibiotic with broad-spectrum antibacterial activity. Levofloxacin acts by inhibiting topoisomerase II or Deoxyribonucleate (DNA) gyrase enzymes in bacteria. This activity instigates a disruption in DNA replication, and ultimately, leads to the bacterial death [1]. Since DNA gyrase enzyme, also present in Mycobacterium tuberculosis, levofloxacin has been included in the management of tuberculosis, as a second-line treatment [2]. For Multi-Drug-Resistant (MDR) patients with tuberculosis, the fluoroquinolone-based regimen is commonly prescribed for nine to eleven months, on average [3].

Since the treatment of MDR TB requires long-term therapy, the use of fluoroquinolone can increase the risk

of unwanted side effects [4]. The serious side effects of fluoroquinolones include liver dysfunction, drug-induced hepatitis, and cholestasis [5]. In addition, fluoroquinolones may also cause kidney failure [6]. It has been reported that approximately 50% of MDR TB patients experience renal disorders, which may progress to end-stage renal disease and mortality [7].

Employing natural resources is likely to be indispensable in efforts to minimize the risk of fluoroquinolones toxicity. Studies have shown that natural resources containing antioxidants are beneficial to reduce nephrotoxicity in animal models [8, 9]. Clove essential oil (*Oleum caryophylli*) is one of the essential compounds that have been widely demonstrated to elicit antioxidant activities [10, 11]. Depending on its origin, clove oil may comprise at least 65 chemical components, with eugenol being 75-83% of the main constituent [11, 12]. Eugenol in clove oil is established to possess free radical scavenging properties, which is more potent than standard antioxidants, such as alpha-tocopherol, and Butylated Hydroxyanisole and Hydroxytoluene (BHA & BHT) formulations [13]. A preliminary study has shown that clove oil treatment could be beneficial to reduce the biomarker levels of liver and kidney damages at a dose of 10 mg/kg in rats induced by the toxic dose of isoniazid-rifampicin [14]. We assumed it would be clinically of significance to explore whether clove oil may also protect renal tissue from levofloxacin toxicity. Toward this putative goal, this study investigated the nephroprotective effect of clove oil on levofloxacin-induced kidney damage in a rat model.

Materials and Methods

Chemicals and drugs: The clove oil used in this study has been standardized and provided with a certificate of analysis (Happy Green®, Indonesia). Levofloxacin generic tablets (500 mg) were purchased from Kimia Farma (Makassar, Indonesia). Curcuma® FCT (Soho, Indonesia) was purchased from a local pharmacy, which each tablet contains 20 mg of *Curcuma xanthorrhiza* rhizome extract. All diagnostic kits for renal biomarkers were purchased from Human Diagnostics Worldwide (Germany), while 1,1,3,3 Tetra-Methoxy-Propane (TMP) solution for Malondialdehyde (MDA) measurement was purchased from Sigma Aldrich (Singapore).

Animals: The experimental animals consisted of 24 male Wistar albino rats (*Rattus norvegicus*) with 200-250 g of body weight. Animals were procured from an animal breeding facility (Yogyakarta, Indonesia). All animals were adapted to laboratory conditions for 7 days prior to initiating the experiments. All experimental pro-

tocols involving animals have been granted an institutional ethical clearance number of UH20050197 under Hasanuddin University.

Thin Layer Chromatography (TLC): The presence of eugenol in the clove oil sample was analyzed using Thin Layer Chromatography (TLC). The clove oil sample and the eugenol standard were compared using a silica gel GF 254 plate as the stationary phase and toluene: acetone (3:1) as the mobile phase. The stains were observed under 254 nm UV light and visualized with an anisaldehyde-sulfuric acid reagent. The presence of eugenol in the clove oil sample is indicated by the presence of a stain that has a similar Retention factor (Rf) value as the standard eugenol compound.

Preparation of clove oil: The multiple oral doses of clove oil that give No Observed Adverse Effect Level (NOAEL) in rats is 200 mg/kg [15]. Consequently, the dose given in this study should not be above the NOAEL. The doses chosen were 10 mg/kg, 25 mg/kg, and 50 mg/kg body weight of rats, which was based on a pilot study [14]. Clove oil was diluted with corn oil to make a solution with a concentration of 0.1%, 0.25%, 0.5% v/v. The clove oil solution was orally administered at a volume of 1 ml/100 g in rats.

Preparation of levofloxacin suspension: The levofloxacin dose used in this study was converted from the highest therapeutic dose in humans (15 mg/kg). Based on a calculation of the conversion of human dose to animal dose by Nair and Jacob [16], the levofloxacin dose for rats is equal to 93 mg/kg. The levofloxacin suspension was prepared by suspending levofloxacin powder in 1% sodium carboxymethyl cellulose and was orally administered at a volume of 1 ml/100g in rats.

Preparation of Curcuma suspension: *Curcuma xanthorrhiza* extract is generally used as a hepatoprotective agent, but several studies have also shown the putative nephroprotective effect of Curcuma extract, and thereby, it was used as a comparison treatment in this study [17]. The animals received a 6.2 mg/kg daily dose of Curcuma extract based on the calculation of the dose conversion of experimental animals [16]

Experimental protocols: The rats were assigned into one of the six groups. A group that did not receive levofloxacin was assigned as the control. The treatment groups were pre-treated with either placebo (corn oil) or clove oil (10 mg/kg, 25 mg/kg, or 50 mg/kg) before administration of levofloxacin suspension. Another group was pre-treated with Curcuma extract as a comparison (posi-

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tive control). The levofloxacin administration was carried out for 28 consecutive days, and the pre-treatments were given 2 hours before levofloxacin on a daily basis. At the end of the experiment, blood samples were withdrawn to measure the levels of renal function biomarkers. Twenty-four hours following blood withdrawal, the rats were euthanized by cervical dislocation and the kidneys were harvested. One kidney was fixed in 10% formalin for histopathological analysis, while the other kidney was frozen using liquid nitrogen for renal MDA assays [18].

Measurement of serum biomarkers: Blood was collected from the lateral vein of rats and stored in EDTA tubes (BD® vacutainer) and centrifuged for 20 minutes at 3000 rpm. The measurement of renal biomarkers is performed as described in a previous study [8]. Serum biomarker analysis was performed using urea and creatinine liquicolor diagnostic kits according to the instructions. The absorbance was measured using a Humalyzer 3500 instrument (Human Diagnostics, Makassar, Indonesia).

Measurement of renal malondialdehyde levels: The MDA levels were measured using the Thiobarbituric Acid Reactive Substances (TBARS) method modified from Adnan et al. study [19]. A total of 400 mg of kidney tissue were homogenized with 2 mL of phosphate buffer (pH 7.4). The mixture was centrifuged for 10 minutes at 3000 rpm. The supernatant (0.5 mL) was collected in a tube and added with 1 mL of 1% thiobarbituric acid and 1 ml of 10% Trichloroacetic acid and heated using a water bath at $100^{o}C$ for 20 minutes. The mixture was centrifuged for another 20 minutes at 3000 rpm to remove precipitates. The absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 531 nm.

Histopathological examination: The kidneys specimens were excised into 0.5-1 cm thick, embedded, and processed in a tissue processor. The specimens were removed and stored in molds and filled with paraffin. The paraffin blocks were sliced (3-4 μm) using a microtome. The tissues were stained using hematoxylin and eosin. Microscopic examination of the renal tissues was carried out using a light microscope (Olympus®) with a magnification of 100× and 400×.

Statistical analyses: The normal distribution of the data was analyzed using Kolmogorov-Smirnov analysis. Normally distributed data were analyzed with one-way ANOVA followed by a Tukey's HSD post-hoc test. If the data distribution is not normal, a Kruskal Wallis and Mann-Whitney test were employed. Significant differences were confirmed when the P-value <0.05.

Results

Thin layer chromatographic analysis of clove oil: Clove oil is known to contain high eugenol compounds. In this study, the clove oil sample was qualitatively analyzed, using Thin Layer Chromatographic (TLC) method and the result confirmed the presence of eugenol compound in the sample as reflected in Figure 1.

Renal malondialdehyde levels: Figure 2 illustrates the amount of renal MDA levels of each group before and after receiving the treatment. It is evident that the group of rats that received levofloxacin and placebo (L+ placebo) for 28 days had the highest level of renal MDA. This indicated that levofloxacin triggered oxidative damage in the rats' kidneys which increased the lipid peroxidation activity as reflected by the high MDA level in the tissue. As seen in Figure 2 , the oral administration of clove oil significantly reduced the renal MDA levels compared to the group that received the placebo treatment. At 10-25 mg/kg doses, the clove oil treatment inhibited the rise in MDA levels as effectively as the Curcuma treatment.

Renal serum biomarkers: The results of the kidney function test, are as presented in Figure 3, demonstrate that the serum urea level markedly increased after administering levofloxacin for 28 days. Administration of clove oil 10, 25, and 50 mg/kg before levofloxacin treatment significantly inhibited the rise in serum urea levels (P<0.05). Even at the lowest dose of 10 mg/kg, the protective effect of clove oil against urea level elevation was seen to be equivalent to that of Curcuma,

Figure 1. Results of thin-layer chromatography

Of clove (a) and standard eugenol (b) oil samples at 245 nm UV light (A) and after spraying of anisaldehyde-sulfuric acid reagent (B).
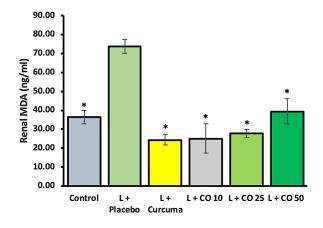
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Figure 2. Renal malondialdehyde levels of rats after administration of levofloxacin and clove oil

L: Levofloxacin; CO: Clove Oil; ** $P<0.01$ versus control; ## $P<0.01$ versus L+placebo.

which was statistically very significant compared to that of the placebo group ($P<0.01$). The serum creatinine of the treatment groups also significantly increased after 28 days of levofloxacin administration. The creatinine levels decrease insignificantly after the administration of low (10 mg/kg) and intermediate (25 mg/kg) doses of clove oil. However, the high dose of clove oil (50 mg/kg) did not prevent the rise in the creatinine levels.

Histopathological examinations: Figure 4 shows the microscopic results of the renal tissue of rats in various treatment groups. The microscopic features of the kidney tissue samples in the normal control group were dominated by normal-looking cells. The glomeruli and tubules had normal appearance, devoid of inflammation and/or cellular degeneration (Figures 4 A-B). Meanwhile, in the levofloxacin plus placebo group, the rat renal tissues exhibited some degrees of fibrosis, necrosis, missing cellular nuclei, and the presence of hyaline cast (pink amorphous mass). Some renal tubules also showed signs of hydropic degeneration (Figures 4, C-D).

In the experimental group that received levofloxacin and clove oil 10 mg/kg, some renal tissue samples exhibited mild hydropic degeneration and necrosis as evident by the presence of hyaline cast in the tubular lumens (Figure 4 E-F). Hydropic degeneration was characterized by the presence of large, pale tubular epithelial cells and clear vacuoles in the cytoplasm. Hydropic degeneration is an early stage of injury and it is considered reversible, which usually occurs due to the inability of the cells to maintain ion and fluid homeostasis. With levofloxacin and clove oil (25 mg/kg) treatment, hemorrhagic areas were also observed in the renal tubules and congestion occurred in the vasculatures, but the glomerular structures did not appear altered (Figure 4 G-H). Meanwhile, the renal tissue of rats that received levofloxacin plus clove oil at a dose of 50 mg/kg exhibited high degrees of renal damage, such as hyaline cast, hydropic degeneration, hemorrhage, and congestion (Figure 4 I-J). However, the damage was less evident than that in the placebo group, where nearly all of the rats had intense renal injury affecting most of the observed areas. Compared to the histopathology observed in the clove oil group, the rats in the Curcuma group exhibited mild renal damage, with
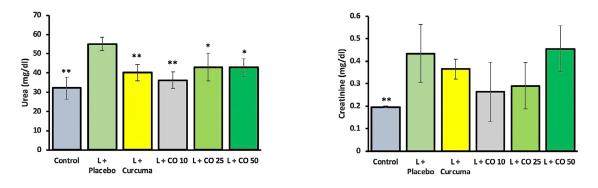

Figure 3. Serum urea and creatinine levels of rats after administration of levofloxacin and clove oil

L: Levofloxacin; CO: Clove Oil; * $P<0.05$ and ** $P<0.01$ versus control; ## $P<0.01$ versus L+placebo.

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hemorrhage and localized inflammatory cells in the examined renal areas (Figure 4 K-L).

Discussion

Long-term use of levofloxacin can cause nephrotoxicity causes oxidative stress. It has been known that glutathione synthesis declines rapidly following levofloxacin administration, leading to the body's inability to create a balance between the production of antioxidants and Reactive Oxygen species (ROS) [20]. The eugenol-like substance that exists in clove oil is a very potent antioxidant compound. Several studies have shown that eugenol can decrease the production of ROS and regulate the antioxidant balance [21-23]. This antioxidant effect is assumed to be capable of protecting against nephrotoxicity, e.g. due to the use of levofloxacin.

Our study results demonstrated that the renal MDA levels of rats treated with clove oil were significantly reduced after they received levofloxacin at a high dose for 28 days. The blood MDA level is an indicator of lipid peroxidation activity; therefore, its reduction suggests the reduced lipid peroxidation in rats treated with clove oil. The mechanism by which eugenol compounds inhibit lipid peroxidation may be in two stages. First, eugenol reduces the production of ROS by inhibiting the formation of oxygen singlets. Second, eugenol is metabolized into dimers and other associated compounds (e.g., diegenol), which further inhibits free radical chain reactions [24].
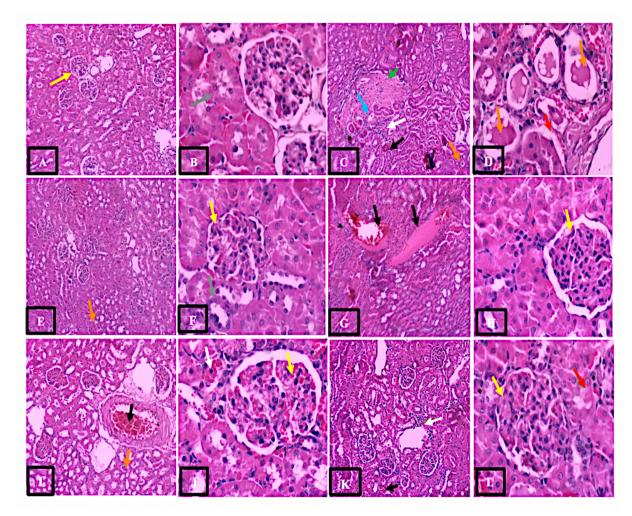

Figure 4. Histological microscopic images of the kidney of rats

In the control group (A, B) and those treated with levofloxacin and placebo (C, D); clove oil 10 mg/kg (E, F), clove oil 25 mg/kg (G, H), clove oil 50 mg/kg (I, J), and curcuma extract (K, L).

Normal glomeruli (yellow arrows) and tubules (gray arrows). Fibrosis (green arrows), hyaline cast (orange arrows), inflammatory areas (white arrows), tubular degeneration (blue arrows), hemorrhage and congestion (black arrows), and hydropic degeneration (red arrows). Photomicrographs were taken at magnifications of 100x (A, C, E, G, I, K) and 400x (B, D, F, H, J, L), respectively.

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In addition to the renal MDA, we also measured the rats' renal function through serum urea and creatinine analysis, which are the widely used serum biomarkers of renal function. The increases in serum urea and creatinine levels indicate acute or chronic renal dysfunction [25]. In this study, the administration of clove oil (10 mg/kg) significantly reduced the elevation of serum urea caused an insignificant decrease in the creatinine level. This occurred partly due to a high standard deviation in the creatinine levels both in groups that received placebo and clove oil. For instance, several animals in the placebo group did not exhibit a marked elevation of creatinine level. It has been argued that a significant increase in creatinine level will only occur when the kidneys have lost at least 50% of their cellular function [26]. In a previous study, it was found that the process to induce renal toxicity was slower than hepatotoxicity in rats [27]. Therefore, it is important to provide a histopathological examination to support the biomarker results, thereby confirming the renal injury in rats.

Upon histopathological analysis, it was found that the damage to the renal tissue occurred most predominantly in the placebo group. The toxic metabolites of Levofloxacin are thought to stimulate renal oxidative stress since it facilitates the ROS formation [20]. The renal damage in the placebo-treated rats was more pronounced in the tubular areas compared to the glomeruli. Other features of renal injury in the placebo group were inflammatory cells infiltration and fibrosis. Tissue fibrosis often occurs when severe injury is accompanied by parenchymal and stromal damages to the tissue, replacing the parenchymal cells with connective tissue cells during recovery [28].

In this study, the clove oil treatment protected both the renal function and structures, especially at a 10 mg/kg dose. The protective effect was comparable to that of Curcuma extract treatment. In clinical settings, Curcuma extract is mostly prescribed as a hepaprotective agent. However, in this study, Curcuma extract was also shown to protect the histopathological structure of the kidney and maintained the renal MDA and serum urea at near-normal levels.

The renal protection of clove oil is thought to be stimulated by eugenol and other antioxidant compounds contained in it. These constituents reduce ROS production and lead to an amelioration of levofloxacin nephrotoxicity in rats, thereby, preventing the acute tubular necrosis [29]. In addition, eugenol may reinforce the body's endogenous antioxidants that are diminished due to levofloxacin administration [30]. It is interesting that the lowest clove oil dose (10 mg/kg) seemed to provide more protection against renal injury than the highest dose (50 mg/kg). Further research is required to investigate the reason why the high doses of clove oil failed to protect against the Levofloxacin toxicity. This might have occurred partly due to eugenol cytotoxicity at its high concentrations [22]. A thorough toxicological study of clove bud extract has been conducted by Vijayasteltar, et al. in 2016 [31]. This study found that the administration of clove bud extract for 90 days at doses up to 1 g/kg did not generate observable clinical signs of injury to the liver or kidneys in Wistar rats. Therefore, it is believed that the oral administration of clove oil may be safe at doses under 1 g/kg in animal models.

Conclusion

Clove oil at a dose of 10 mg/kg reduced the nephrotoxicity of levofloxacin based on the serum levels of creatinine, urea, and the renal MDA analysis. In addition, clove oil at a dose of 10 mg/kg was also found to lessen renal histopathological injury, which was otherwise intense and diffused in the levofloxacin rats treated with placebo only. The protection provided by 10 mg/kg of clove oil and Curcuma extract was similar. Therefore, it is assumed that clove oil might potentially be used as a nephroprotective agent against kidney damage due to the chronic use of levofloxacin.

Limitations of the study: The clove oil nephroprotective effect is believed to occur due to its antioxidant properties. However, this study did not measure the levels of antioxidant enzymes at the end of the experiments to confirm a potential antioxidant-related mechanism for the nephroprotective effect of clove oil.

Recommendations for future research: We recommend that in further studies the levels of endogenous antioxidants be explore in animal models. To verify the nephroprotective effect of clove oil, potential enzymes to be investigated following clove oil treatment may include superoxide dismutase, glutathione peroxidase and catalase.

Ethical Considerations

Compliance with ethical guidelines

All experimental protocols involving animals have been granted institutional ethical clearance number of UH20050197 under Faculty of Medicine, Hasanuddin University.

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